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Experimental infection of wild boar and domestic pigs with a Foot and mouth disease virus strain detected in the southeast of Bulgaria in December of 2010

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ABSTRACT

Foot and mouth disease (FMD) was detected in a wild boar in Southeastern Bulgaria in December 2010. The occurrence and spread of the disease in wild cloven-hoofed animals may pose an unexpected and significant threat to FMD virus (FMDV)-free areas within and outside the European Union. So far, only one well documented experimental infection with FMD in wild boar has been published. In order to obtain more epidemiologically relevant data regarding the disease in wild boar we conducted an experiment with the 2010 Bulgarian FMDV type O isolate. Two young wild boar were challenged while two domestic pigs and two additional wild boar served as contact controls. While the domestic pigs developed severe clinical signs of FMD, the wild boar showed relatively mild course of the disease. Viremia started in contact wild boar 2 days post exposure (DPE) and lasted until 6 DPE. The virus shedding lasted until 9 DPE. On 27 DPE, when the animals were slaughtered, viral RNA was detected in lymphoid tissues and oropharyngeal fluid but no virus could be isolated. Commercial ELISAs and virus neutralisation tests detected antibodies against FMDV on 8 or 6 DPE, respectively.

The data of the present study will help to understand FMD in wild boar populations and can be used in models to evaluate the potential role of wild boar in FMD epidemiology.

1. Introduction

Shortly after Foot and mouth disease (FMD) had been detected in a wild boar in southeastern Bulgaria in December 2010 close to the Turkish border, clinical cases in susceptible farmed animals were confirmed in several villages nearby. In March 2011, 5 out of 11 wild boar were found seropositive in Turkey, on the other side of the border. The VP1-sequence of the Bulgarian wild boar isolate (Valdazo-Gonzalez et al., submitted for publication) showed only one nucleotide change compared to Turkish Foot and mouth disease virus (FMDV) Type O isolates of the PanAsia-2ANT-10 lineage, isolated in July 2010 at the Turkish Eastern Black Sea coast (Valdazo-Gonzalez et al., 2011). The extent of infection in the wild boar population and the duration of the sylvatic epidemic are unknown. However, FMD in wild boar may pose an unexpected but significant threat to the whole European Union and other FMDV-free areas with a relevant wild boar or feral pig population. While wild boar was not an important reservoir for FMD in the past, there are two reasons to reassess it is potential role. Since the 1950s, populations of wild boar increased (Apollonio et al., 2010). Based on hunting statistics, Germany and France are believed to

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have among the highest population densities of wild boar in Europe, followed by Spain, Poland and the Czech Republic (Müller et al., 2011). Furthermore, as viruses change constantly and the isolation of FMDV from wild boar in Bulgaria was quite surprising, the new strain may show unexpected properties in this species. In contrast to previous assumptions, FMD in wild boar may pose an unexpected but significant threat to the whole European Union and other FMDV-free areas with a relevant wild boar or feral pig population. An assessment of this threat requires data on susceptibility, incubation period, clinical course, viremia, virus shedding, immune response and potential carrier state of wild boar. Only one well documented FMDV infection experiment in this species has been published (Mohamed et al., 2011) while some other reports do not provide much details (Ercegovac et al., 1968; Yadin and Chai, 1994). In order to obtain more data on the recent 2010 Bulgarian FMD isolate in wild boar we therefore conducted an experiment with this FMDV type O isolate.

2. Materials and methods

Two 4-month-old captive wild boar (8–15 kg), obtained from a zoological garden, were experimentally inoculated in the bulb of the heel with the virus isolated from a wild boar in Bulgaria (O/BUL/1/2010) and obtained from the Bulgarian National Veterinary Service. The animals received 0.2 ml of the 2nd IBRS-2 (CCL-RIE 103) cell culture passage containing $10^{6.5}$ tissue culture infectious dose ($TCID_{50}$) of FMDV. One day later, two wild boar of the same age and two domestic pigs (8 weeks old, about 15 kg) were placed into the same stable and were allowed to have free contact with the inoculated wild boar. Body temperatures and clinical signs were recorded daily during the duration of the experiment (4 weeks). Blood samples were taken on 1–5, 7, 9, 12, 15, 18, 21, and 28 days post infection (DPI), nasal and saliva swabs were taken daily (in 1.5 ml cell culture medium, 1–24 DPI and 28 DPI) for titration, virus isolation and detection of FMDV RNA. At 28 DPI, all animals were euthanized and several tissue and probang (oropharyngeal fluid, OPF) samples were taken for virus isolation and detection of FMDV RNA. Virus titres were assayed in fetal goat tongue cells (CCLV-RIE 127) (Brehm et al., 2009) and titres were calculated by end-point titration according to Spearman–Kaerber method (Kaerber, 1931; Spearman, 1908). Virus isolation was performed on fetal goat tongue cells for 96 h using 100 μl serum or supernatant of swab samples and homogenized tissue samples (pea-sized, homogenized in 1 ml cell culture media). Probang samples were prediluted (1:3) in cell culture media. The viral RNA load was determined by using a FMDV specific real-time reverse transcription PCR (Moniwa et al., 2007). Antibody detection in serum (0, 5, 7, 9, 12, 15, 18, 21, 28 DPI) was carried out using the PrioCHECK® FMDV NS and PrioCHECK® FMDV Type O according to the manufacturer's instructions (Prionics). Sera were tested in a virus neutralization test (VNT). The test was performed according to the OIE manual (OIE, 2004). For all VNTs, FMDV O1 Manisa was used for titration on BHK21 cells (CCLV-RIE 164).

The experiment was conducted under biosaftey level 3+ conditions. The animal experiment was reviewed and approved by the responsible state ethics animal welfare committee (trial approval LVL M-V/TSD/7221.3-3.2-001/11).

3. Results

3.1. Clinical signs

First clinical signs were noticed in the domestic contact pigs as early as 2 days post exposure (DPE). Both animals had fever (41.2 °C), poor appetite and showed limping and swelling of the coronary band. Vesicles appeared on the snout and the digits. On 3 DPE pronounced symptoms were seen with swelling and reddening of the coronary band and numerous ruptured and unruptured vesicles affecting every foot and the snout. The domestic pigs had to be euthanized due to the severe clinical impairment.

In contrast, first symptoms in wild boar were seen on 4 days post infection (DPI) and 4 DPE, respectively. The lesions started with vesicles on the dorsum of the snout (Fig. 1a) and the interdigital space (Fig. 1b). During the next days several ruptured vesicles in the interdigital space, the coronary band, the digits, the heel, claws and the lips (Fig. 1c and d) with serofibrinous in-filling were detectable (Fig. 1e). 28 DPI healing and claw deformation (Fig. 1f) was visible. Food intake and activity were considered almost normal and lameness was not observed. In both contact wild boar, increased body temperature (>40.0 °C) was measured between 3 and 5 DPE. The temperature curve is given in detail in Fig. 2a–f.

3.2. Detection of viral RNA and virus isolation

The virological results of individual serum and swab samples are shown in Fig. 2a–f. Table 1 shows the results of virus isolation and PCR of tissue samples and OPF.

3.2.1. Serum samples

In contact domestic pigs viremia started on 2 DPE and lasted until death (4 DPE). Viral RNA in serum was detected up to 4 DPE. The maximum viral titre in sera of domestic contact pigs was $10^{5.50}$ $TCID_{50}$ per ml (3 DPE).

Viremia was detected in contact wild boar on 2 DPE and lasted until 6 DPE whereas viral RNA in serum was found until 8 DPE. Viral titres up to $10^{7.0}$ $TCID_{50}$ per ml (3 DPE) were verified in serum samples.

In infected wild boar viremia started 1 DPI and lasted until 9 DPI, viral RNA was detected up to 18 DPI. Serum viral titres up to $10^{7.0}$ $TCID_{50}$ per ml (2 DPI) were found.

3.2.2. Nasal and saliva swab samples

In general, viral RNA was detectable 1 DPI/DPE until 24 DPI/DPE. During the first week, nasal swabs generally yielded lower viral RNA loads compared to saliva swabs, hence virus isolation was performed on both types of samples, but titration was only done on saliva samples.

In needle infected wild boar, the peak of viral RNA load was found 3 DPI in saliva (ct 19.98) and 4 DPI in nasal

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Fig. 1. (a–e) Lesions after FMDV type O infection of wild boar. Vesicles on the dorsum of the snout (a) and the interdigital space (b), 4 DPI. Ruptured vesicles on the heel 8 DPI (c) and 28 DPI (d). Serofibrinous infilling in the interdigital space, 8 DPI (e). Claw deformation after coronary band lesions, 28 DPI (f).

swabs (ct 26.31). Viral RNA was found intermittently beyond 13 DPI and was detectable until 24 DPI. Infectious virus was isolated until 12 DPI. Virus titres in saliva swabs peaked 3 DPI with $10^{5.25}$ $TCID_{50}$ per ml.

The maximum amounts of viral RNA in contact wild boar were detected 3 DPE in saliva (ct 16.27) and nasal swabs (ct 25.35). Detection of viral RNA was intermittent beyond 13 DPE and the last positive sample was found on 23 DPE. Infectious FMDV was detected until 9 DPE. Virus titres in saliva swabs peaked 3 DPE with $10^{5.75}$ $TCID_{50}$ per ml.

In contact domestic pigs the highest amounts of viral RNA were found on 2 DPE in saliva (ct 25.24) and nasal swabs (ct 15.95). Infectious virus and viral RNA were found until euthanasia (4 DPE) with a maximum virus titre on 3 DPE with $10^{3.25}$ $TCID_{50}$ per ml.

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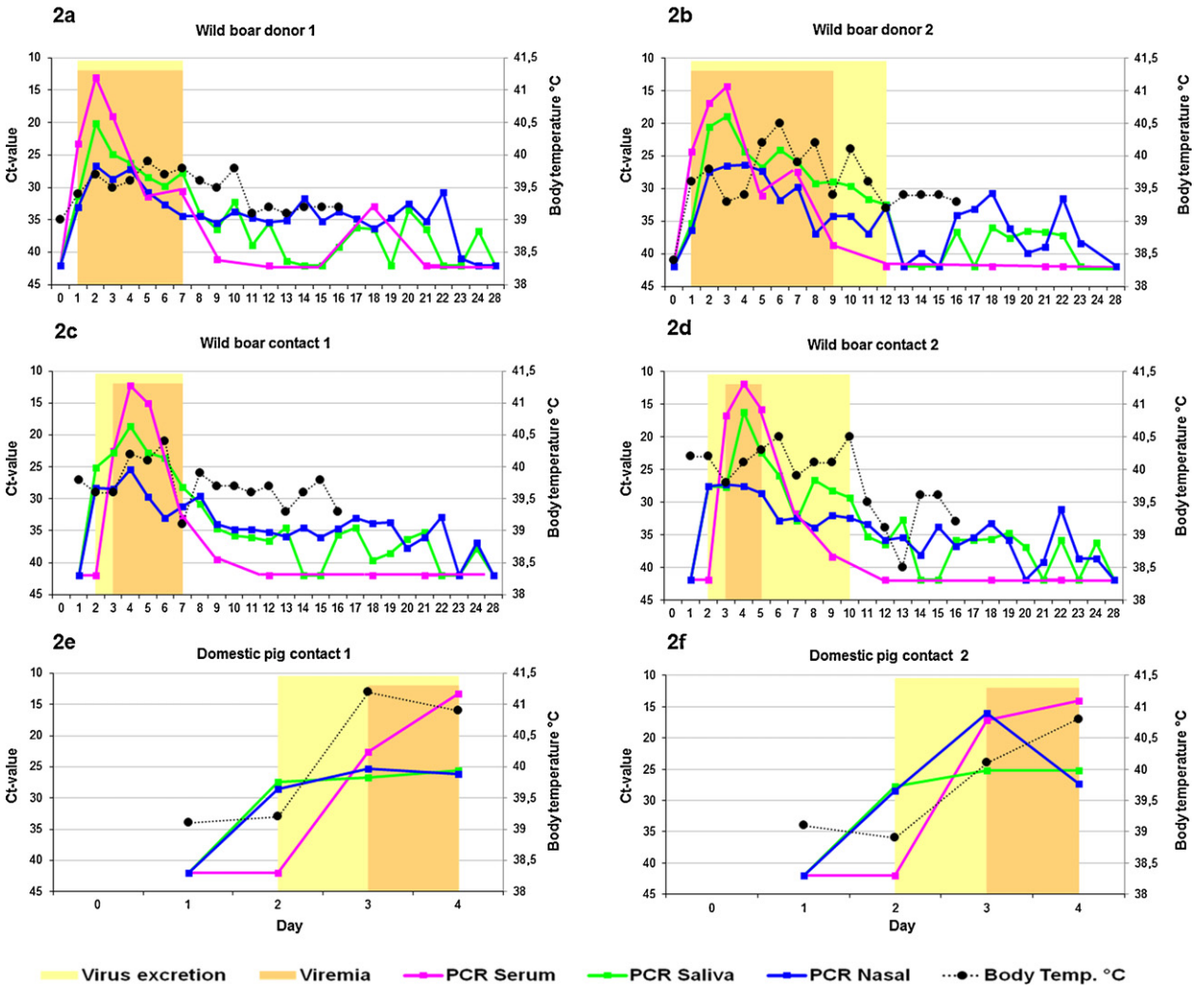

Fig. 2. (a–f) Viral RNA load, virus isolation results and body temperature of FMDV infected and contact wild boar and contact domestic pigs. Virus isolation data of saliva swab (virus excretion) and serum samples (viremia) are shown in yellow and orange reflecting the time period when a cytopathogenic effect was seen. Viral RNA detected in swab samples and serum by rRT-PCR are shown in pink, green, and blue lines (ct values referring to the left axis). The body temperature (black line) is given in °C. The time (*x*-axis) is given in days, equal to days post infection (DPI). For contact animals DPI-1 is equivalent to days post exposure (DPE).

3.2.3. Oropharyngeal fluid (OPF) and tissue samples

On 28 DPI (27 DPE, day of slaughter), viral RNA was detected in affected skin areas (snout, bulb of the heel), the soft palate and lymphoid tissues (tonsil, tracheobronchial and retropharyngeal lymph nodes) and also in oropharyngeal fluid. However, no virus could be isolated from these samples.

3.3. Serology

Antibodies could be detected by commercial ELISAs (Priocheck® FMDV-NS and Priocheck® FMDV Type O) on 7 DPI and 8 DPE, respectively. Neutralizing antibodies were detected by VNT on 5 DPI and 7 DPI in donor wild boar and on 6 DPE and 8 DPE in contact wild boar, respectively (Fig. 3).

4. Discussion and conclusions

The most striking result of the experiment described is the discrepancy in the clinical course of FMD between wild boar and domestic pigs. While the domestic pigs had to be euthanized due to severe clinical FMD, the general condition of wild boar was less affected. Although wild boar displayed severe foot lesions, the animals' mobility did not appear to be impaired. These finding suggest that infected wild boar may survive the disease, stay mobile and excrete FMD virus over a longer period, in our experiment up to 9 days after contact infection.

The incubation periods were 2 days for domestic pigs and 4 days for wild boar.

While also Mohamed et al. (2011) noted that feral swine exhibited a higher tolerance for FMD compared to

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Table 1
Virus isolation and rRT-PCR results of tissue samples and OPF after FMDV infection of wild boar, 28 DPI (27 DPE).

Sample	rRT-PCR ct value/virus isolation							
	WB donor 1*		WB donor 2		WB contact 1		WB contact 2	
	ct value	VI†	ct value	VI	ct value	VI	ct value	VI
OPF (probang)	32.67	–	32.75	–	32.48	–	34.63	–
Snout	33.23	–	33.13	–	35.77	–	36.66	–
Tonsil	31.96	–	30.86	–	30.46	–	29.80	–
Palate, soft	32.72	–	34.46	–	34.08	–	30.35	–
Retropharyngeal ln.‡	33.83	–	31.16	–	31.18	–	29.66	–
Tracheobronchial ln.	36.55	–	33.39	–	33.91	–	30.47	–
Lung, main lobe	No Ct	–	No Ct	–	39.22	–	No Ct	–
Nasal mucosa	No Ct	–	No Ct	–	No Ct	–	No Ct	–
Skin, bulb of the heel	36.81	–	37.95	–	33.17	–	No Ct	–
Musculature	No Ct	–	No Ct	–	No Ct	–	No Ct	–

* WB: wild boar.
† VI: no cytopathogenic effect (−), cytopathogenic effect (+).
‡ ln.: lymphnode.

domestic pigs, they observed not only transient fever and vesicular lesions but also lameness in feral swine. In their experiment, clinical scores of feral and domestic swine were comparable and in contrast to our study, two contact feral swine had to be euthanized due to severe lameness and inability to stand (Mohamed et al., 2011). Furthermore, they observed an incubation period in feral swine of only 2 days.

There are several factors that have to be considered when evaluating these discrepancies. Certainly, age and size of the experimental animals can influence the clinical course of disease, in particular when the feet are affected. Also the different viruses (A24 Cruzeiro vs. O Bulgaria 2010) used in the experiments may have had an effect on the clinical signs. A striking example for the importance of the virus strain was seen in the FMDV type O epizootic in Taiwan 1997 when only pigs but no cattle showed clinical signs (Pacheco and Mason, 2010). While we do not assume that the two passages of the O Bulgaria 2010 isolate (obtained from Bulgarian wild boar) in IB-RS-2 cells, a line originating from pigs, had an effect, also this step, which was necessary to obtain a sufficient amount of virus for the study, has to be mentioned. Furthermore, the virus dose may have an influence on the incubation period.

In the present study the wild boar started to shed virus 1 day after infection or contact without showing any clinical signs. Thus, it was again demonstrated that transmission can occur before the onset of clinical signs

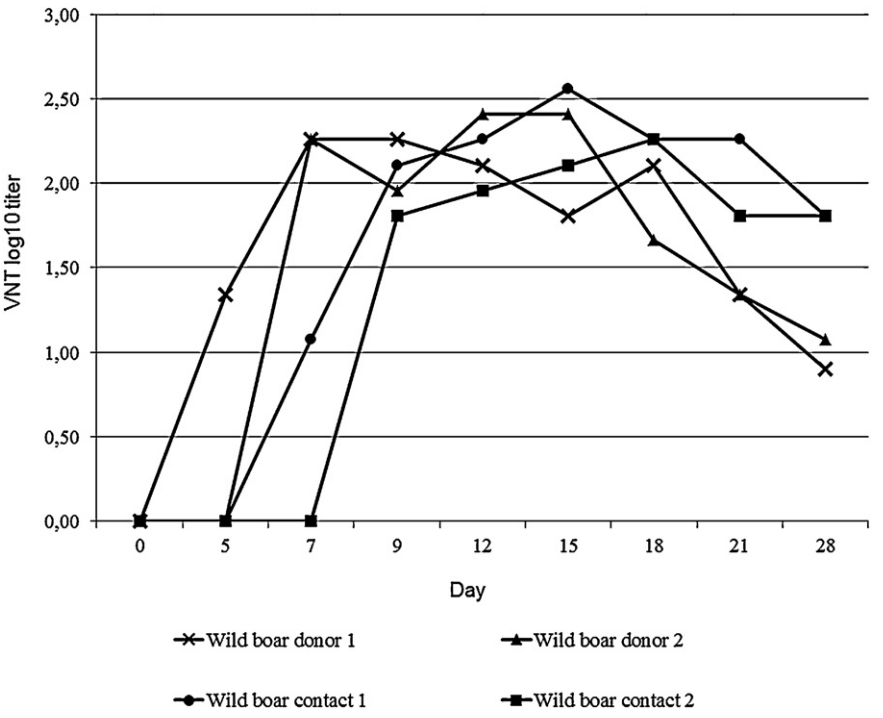

Fig. 3. Virus neutralization titre. Sera were tested in virus neutralization tests (VNT) according to the OIE manual. FMDV O1 Manisa was used for titration on BHK21 cells. The time (*x*-axis) is given in days, equal to days post infection (DPI). For contact animals DPI-1 is equivalent to days post exposure (DPE).

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which was discussed recently by Orsel et al. (2009) who pointed out that after clinical recognition of FMD, priority should be given to trace back contacts with swine and dairy farms, as they may already have been infectious in the incubation period (Orsel et al., 2009). In contrast, Charleston et al. (2011) observed transmission in cattle only after the onset of clinical signs, but this may have been due to the experimental setup and the species used (Charleston et al., 2011). With regard to wild boar being affected, retrospective analysis of potential contact animals is impossible. Taking into account that the mobility of the animals may not be significantly impaired by the disease, we consider the early onset of viral excretion as further evidence for the potential role of wild boar for the spread of FMD, in particular in areas with a high wild boar population density.

Also in respect to the detection of virus specific RNA, there are differences between our study with O Bulgaria 2010 and the study done with A24 Cruzeiro. Whereas in the A24 Cruzeiro experiments, FMDV specific RNA was detectable only intermittently in the oral swabs of feral swine between 1 and 8 DPI/DPE and not beyond 14 DPI/12 DPE (Mohamed et al., 2011), in our study FMDV specific RNA was found constantly until 13 DPI/DPE and intermittently until 23 DPE and 24 DPI. Again, the age of the animals as well as the viral strain and dose may have played role, but also the sensitivity of the assays and RNA isolation protocols may have been different.

Although it is generally assumed that pigs do not become virus carriers (FMDV carriers are defined as animals being virus positive for at least 28 DPI), some reports have pointed out the presence of FMDV RNA in blood for a period of at least 28 DPI (Mezencio et al., 1999). In our study, viral RNA but no infectious virus was detected in tissue samples and OPF. Also in the A24 Cruzeiro experiment, FMDV specific RNA persisted in the tonsils up to 33–36 DPI/DPE, whereas virus isolation was negative (Mohamed et al., 2011). Based on these findings and the lack of evidence for a carrier state in domestic pigs, we assume that wild boar do probably not play a crucial role as virus carriers. However, at this time point, a conclusively analysis of the carrier status should not be made, as it is important to note that the lacking detection of infectious virus in tissues and OPF might be due to the presence of neutralizing antibodies or an insufficient sensitivity of the fetal goat tongue cell line. Based on our data we cannot exclude that replication can occur later on.

In addition to ELISAs, virus neutralisation tests (VNT) were performed to evaluate the kinetics of neutralizing antibodies. Remarkably, the titre of neutralizing antibody peaked between days 9 and 18 and declined until 28 DPI (27 DPE). The switch from IgM to IgG antibody may explain this observation of some extent. After needle and contact infection of domestic pigs with FMDV O1 Manisa, IgM antibodies were secreted after 7 DPI (peak, 7 DPI) and declined until 28 DPI, whereas IgG antibodies were detectable 14 DPI and peaked on 28 DPI (Pacheco et al., 2010). We assume the antibody peak observed in our study by VNT is due to IgM secretion. Whether neutralizing antibodies in wild boar generally decline as quickly as observed here, reaching levels around the threshold of detection already at 28 DPI remains unclear.

The present data indicate that wild boar do not play an important role as virus carriers. The clinical, virological and serological data generated in our FMD experiment will be helpful for future models to further evaluate the potential role of wild boar in FMD epidemiology.

Conflict of interest

The authors affirm that no financial or personal relationships existed that could have inappropriately influenced the content of this manuscript or the opinions expressed.

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